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Taming the neutrophil: calcium clearance and influx mechanisms as novel targets for pharmacological control

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Summary

Neutrophils are relatively insensitive to the anti-inflammatory actions of conventional chemotherapeutic agents, including corticosteroids, emphasizing the requirement for novel pharmacological strategies to control the potentially harmful proinflammatory activities of these cells. In the case of commonly-occurring inflammatory diseases of the airways, the neutrophil is the primary mediator of inflammation in conditions such as chronic obstructive pulmonary disease, cystic fibrosis, acute respiratory distress syndrome, bronchiectasis and non-eosinophilic bronchial asthma. Recent insights into the mechanisms utilized by neutrophils to restore Ca^{2+} homeostasis following activation with Ca^{2+}-mobilizing, proinflammatory stimuli have facilitated the identification of novel targets for anti-inflammatory chemotherapy in these cells. The most amenable of these from a chemotherapeutic perspective, is the cyclic AMP-dependent protein kinase-modulated endomembrane Ca^{2+}-ATPase which promotes clearance of the cation from the cytosol of activated neutrophils. Second generation type 4 phosphodiesterase inhibitors and adenosine receptor agonists operative at the level of subtype A2A adenosine receptors, which are currently undergoing clinical and preclinical assessment respectively, hold promise as pharmacologic modulators during the restoration of Ca^{2+} homeostasis. If this promise is realized, it may result in novel chemotherapeutic strategies for the control of hyperacute and chronic inflammatory conditions in which neutrophils are primary offenders. Alternative, potential future targets include the Na^{+}, Ca^{2+}-exchanger and store-operated Ca^{2+} channels, which cooperate in the refilling of intracellular Ca^{2+} stores.

Keywords: calcium clearance, calcium influx mechanisms, neutrophils, pharmacological control

Introduction

The destructive power of activated neutrophils is well recognized, with neutrophil-mediated tissue injury contributing significantly to the pathogenesis of numerous diseases. This review is focused on Ca^{2+} handling by activated neutrophils, with special emphasis on our current understanding of restoration of Ca^{2+} homeostasis, as well as emerging anti-inflammatory strategies targeting Ca^{2+} fluxes in activated neutrophils.

Neutrophil-mediated tissue injury: reasons for concern

The destructive power of activated neutrophils

The crucial involvement of polymorphonuclear leucocytes, predominantly neutrophils, in host defence is underscored by the relative abundance of these cells, with approximately 50 billion circulating in the bloodstream [1]. Following extra-vasation, neutrophils attracted to sites of tissue inflam-

Table 1. Examples of clinical disorders associated with neutrophil-mediated tissue injury and organ dysfunction classified according to the predominant system involved.

Respiratory system	Acute respiratory distress syndrome
	Bronchiectasis
	Cystic fibrosis
	Chronic obstructive pulmonary disease
	Idiopathic pulmonary fibrosis
	Hypersensitivity pneumonitis
	Pulmonary vasculitides
	Non-eosinophilic bronchial asthma
	Goodpasture's syndrome
	Wegener's granulomatosis
	Silicosis
Cardiovascular system	Systemic inflammatory response and multi–organ dysfunction syndromes
	Immune complex-mediated vasculitides of infective and non-infective origin
	Acute coronary syndromes
	Ischaemia-reperfusion injury
	Endocarditis
Genitourinary tract	Immune complex-mediated glomerulonephritis of both infective and non-infective origin
	Pyelonephritis
Gastrointestinal tract	Inflammatory bowel disorders
	Chronic active gastritis
Musculo-skeletal system	Osteomyelitis
	Rheumatoid arthritis
	Gout
	Articular chrondrocalcinosis

mation by chemotaxins internalize microbial pathogens. Inside the phagosome these are targeted and destroyed by toxic reactive oxidants and serine proteases released into the phagolysosome. Production and/or release of these toxic oxidants and proteases by neutrophils is stringently controlled to protect surrounding cells and tissues [2], but may be excessive as a result of inappropriate activation and necrosis. Excessive and/or protracted activation of neutrophils during hyperacute and chronic inflammatory disorders predisposes to tissue injury. Important examples of inflammatory conditions in which the neutrophil is the primary offender are shown in Table 1.

Notwithstanding the cardiovascular system, the predominance of inflammatory airways disorders shown in Table 1 demonstrates the susceptibility of the lung to neutrophil-mediated injury. The lung is clearly a primary target for neutrophil recruitment and activation, which in turn contributes to the pathogenesis of conditions such as chronic obstructive pulmonary disease (COPD) [3], asthma [4] and cystic fibrosis [5].

Corticosteroids and neutrophils

Few currently available therapeutic agents, including corticosteroids, effectively down-regulate neutrophil pro-inflammatory activity. Insensitivity to corticosteroids may therefore be a feature of those disorders in which the neutrophil is the predominant inflammatory cell type.

The relative insensitivity of neutrophils to corticosteroids is attributable to a combination of mechanisms. First, many of the proinflammatory activities of these cells [production of reactive oxidants, release of granule polypeptides, generation of prostanoids, eicosanoids and platelet activating factor (PAF)] occur within seconds of activation and are independent of *de novo* protein synthesis. Secondly, neutrophils which are now recognized to be an important source of newly synthesized cytokines [6,7], particularly interleukin (IL)-8 and tumour necrosis factor (TNF)-α, contain comparatively high levels of the functionally inactive beta isoform of the glucocorticoid receptor (GR), the synthesis of which is further up-regulated on exposure of the cells to IL-8 [8], rendering them even less corticosteroid-sensitive. Moreover, neutrophils, unlike other types of immune and inflammatory cells, have been reported to be relatively insensitive to the apoptosis-inducing actions of corticosteroids [9,10].

Clearly, the design and development of novel, neutrophil-directed anti-inflammatory, chemotherapeutic strategies is a priority.

Calcium and neutrophils

Receptor-mediated transient increases in cytosolic Ca^{2+} precede, and are a prerequisite for the activation of the proinflammatory activities of neutrophils. Ca^{2+}-dependent functions include activation of the membrane-associated superoxide-generating electron-transporter, NADPH oxidase, adhesion to vascular endothelium, degranulation, activation of phospholipase A_2 and synthesis of IL-8. Because of this critical dependence of activation of the proinflammatory activities of neutrophils on Ca^{2+}, the mechanisms utilized by these cells to both mobilize and dispose of the cation have been identified as potential targets for anti-inflammatory chemotherapy.

Calcium handling by activated neutrophils

Mobilization of Ca^{2+}

Intracellular Ca^{2+} in neutrophils is reportedly stored in specialized storage vesicles termed calciosomes [11]. This may, however, be somewhat of an oversimplification as there appear to be at least two distinct cellular locations for Ca^{2+} stores in neutrophils that may have differential involvement in activation of proinflammatory functions, and may utilize different molecular/biochemical mechanisms of Ca^{2+} mobilization [12]. One site is located peripherally under the

plasma membrane and appears to be involved in the activation of β_2-integrins, while the other is localized in the perinuclear space and is mobilized by chemoattractants such as the synthetic tripeptide, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) [12]. Mitochondria may also serve as calcium-storage organelles [13], with neutrophils possessing a more extensive mitochondrial network than previously recognized [14].

The molecular/biochemical mechanisms involved in Ca^{2+} mobilization following chemoattractant receptor-mediated activation of neutrophils are well characterized. Leucocyte membrane receptors for chemoattractants, including FMLP, C5a, leukotriene B_4, PAF and chemokines, belong to the 7-transmembrane, G-protein-coupled family of receptors. Occupation of these receptors, which are controlled by various $G\alpha$ and $G\beta\gamma$ subunits, results in activation of the β isoforms of phospholipase C which in turn mediate production of inositol-1,4,5-triphosphate (IP_3) by hydrolysis of phosphatidylinositol 4,5 biphosphate [15,16]. IP_3 interacts with Ca^{2+}-mobilizing receptors on intracellular storage vesicles, resulting in discharge of the cation into the cytosol. These events are extremely rapid, occurring within a few seconds of ligand-receptor binding, and result in a five to 10-fold increase in the cytosolic free Ca^{2+} concentration above a basal value of about 100 nM [17]. Only modest increases in IP_3, of around 15% of maximal, are required to cause complete mobilization of intracellular Ca^{2+} [18,19]. The peak increase in cytosolic Ca^{2+} is followed by a rapid, progressive decline in cytosolic Ca^{2+} with a return to basal values within several minutes. The rate of decline in the concentration of cytosolic Ca^{2+} appears to be governed by two mechanisms. First, the efficiency of the systems which promote clearance of Ca^{2+} from the cytosol [20,21] and secondly, those which regulate the time of onset, rate and magnitude of influx of extracellular cation [22].

Clearance of Ca^{2+} from the cytosol of activated neutrophils

Following activation of neutrophils, restoration of Ca^{2+} homeostasis is essential to prevent Ca^{2+} overload and hyperactivity of the cells. This is achieved by rapid clearance of Ca^{2+}, primarily through the action of two adenosine triphosphate (ATP)-driven pumps operating in unison. These are the plasma membrane Ca^{2+}-ATPase, which is a Ca^{2+}-efflux pump, and the endo-membrane Ca^{2+}-ATPase which resequesters the cation into intracellular stores [23]. In activated neutrophils, these two Ca^{2+} pumps appear to contribute more or less equally to the clearance of cytosolic Ca^{2+} [24,25].

The plasma membrane Ca^{2+}-ATPase of neutrophils is modulated by calmodulin, acidic phospholipids and polyunsaturated fatty acids which shift the pump to a higher-affinity state for Ca^{2+}, resulting in enhanced maximal velocity [26]. In the case of FMLP-activated neutrophils, a dramatic and transient efflux of Ca^{2+} immediately follows release of the cation from stores, proceeds over a 30-s time course and results in discharge of about 50% of Ca^{2+} mobilized from stores [24,25].

Activation of neutrophils with FMLP also results in immediate, transient activation of adenylate cyclase [27,28]. This results from the interaction of adenosine, generated by dephosphorylation of adenylates, with G-protein/adenylyl cyclase-coupled adenosine receptors (AR) of the A_{2A} receptor subtype on the neutrophil membrane [27,28], leading to activation of adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase A (PKA). PKA in turn up-regulates the Ca^{2+} sequestering/resequestering activity of the endomembrane Ca^{2+}-ATPase by phosphorylation of the regulatory polypeptide, phospholamban [29]. The endomembrane Ca^{2+}-ATPase contributes to Ca^{2+} clearance from the cytosol of activated neutrophils by promoting resequestration of cation mobilized from stores, as well as sequestration of incoming Ca^{2+} during store-operated influx [30–32].

Operating in harmony, these two Ca^{2+} pumps (plasma membrane and endomembrane) appear to be the primary contributors to clearance of cytosolic Ca^{2+} in activated neutrophils.

The role of membrane depolarization

Efficient clearance of Ca^{2+} by the plasma membrane and endomembrane Ca^{2+}-ATPases is facilitated by the membrane depolarizing action of NADPH oxidase which limits the influx of extracellular Ca^{2+}. NADPH oxidase is the membrane-associated, electron-transporting, superoxide-generating system of phagocytes, which undergoes Ca^{2+}-dependent activation during exposure of the cells to chemoattractants, cytokines and opsonized antigens. The dramatic decrease in membrane potential that accompanies activation of NADPH oxidase, and which is coincident with release of Ca^{2+} from neutrophil intracellular stores, efflux of the cation and activation of superoxide production, has been attributed to the electrogenic activity of the oxidase [22], as well as to the action of a rapidly activated H^+ conductance with resultant influx of H^+ [33]. This type of abruptly occurring membrane depolarization has been shown to limit the influx of extracellular Ca^{2+} [34,35]. When the cells are depolarized, the driving force for entry of Ca^{2+} is abolished because the electrical component of the electrochemical gradient promoting Ca^{2+} entry is markedly reduced.

Membrane repolarization and store-operated influx of Ca^{2+}

The uptake of extracellular Ca^{2+} into FMLP-activated neutrophils is a delayed event, with net influx becoming detectable only at around 1 min after addition of the chemoattractant, and proceeding gradually over a 5-min time-course. This type of Ca^{2+} influx is characteristic of

store-operated mechanisms, being secondary to emptying of intracellular Ca^{2+} stores and necessary for store refilling.

Interestingly, the time-course of influx of extracellular Ca^{2+} is superimposable on that of membrane repolarization, suggestive of a mechanistic interrelationship between these two events. It has been proposed that membrane repolarization in activated eosinophils is mediated by an NADPH oxidase-associated H^+ extrusion channel which, being electrogenic, results in repolarization of the cell membrane [33,36].

Notwithstanding the crucial role of proton efflux in the regulation of intracellular pH, additional mechanisms may contribute to membrane repolarization in activated neutrophils. Importantly, charge compensatory mechanisms may vary according to the nature of the signal transduction pathways utilized. Membrane repolarization in chemoattractant-activated neutrophils is associated with up-regulation of the activity of the electrogenic plasma membrane Na^+/Ca^{2+} exchanger operating in reverse mode [37].

Treatment of activated neutrophils with KB-R7943, a selective inhibitor of the Na^+/Ca^{2+} exchanger operating in reverse mode [38], significantly inhibited the rate and magnitude of recovery of the membrane potential towards resting levels (Fig. 1). Importantly, inhibition of membrane repolarization by KB-R7943 is associated with marked attenuation of store-operated Ca^{2+} uptake by these cells, suggesting that these processes are interdependent [37].

The role of membrane repolarization in regulating the rate of calcium influx is further supported by the observation that addition of the NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI) to neutrophils 1 min after FMLP potentiated the rate and magnitude of repolarization (Fig. 1), with a corresponding increased rate of store-operated Ca^{2+} influx [37].

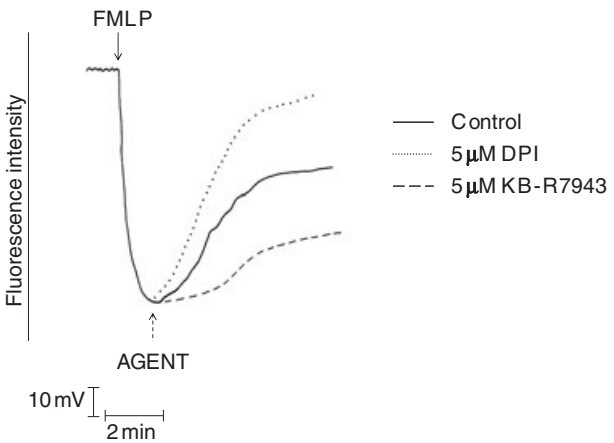

Fig. 1. The effects of KB-R7943 (5 μM) and DPI (5 μM) on the N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (1 μM)-activated (↓) membrane repolarization responses of human neutrophils. The test agents were added to the cells 1 min after FMLP (↑) at the time of maximal depolarization.

Recently, high conductance Ca^{2+}-activated K^+ channels have been elegantly demonstrated to participate in charge compensation following activation of neutrophils with phorbol esters and opsonized microorganisms. The resultant electrogenic K^+ efflux (influx in the case of phagocytic vacuoles) is a prerequisite for intravacuolar activation of antimicrobial proteases [39,40].

Store-operated Ca^{2+} channels and neutrophils

Although the precise molecular identity of the store-operated Ca^{2+} channels (SOCs) operative in human neutrophils and other cell types has not been conclusively established, one particular family of non-voltage-activated Ca^{2+} channels, the family of transient receptor potential (TRP) channels has attracted considerable interest. These channels have been the subject of several recent reviews [41,42], and their salient features with respect to neutrophils can be summarized as follows:

- There are three major subgroups within the TRP gene family, the proposed nomenclature for these being TRPC, TRPV and TRPM, each of which currently contains seven (TRPC 1, 2, 3, 4, 5, 6, 7) five (TRPV 1, 2, 4, 5, 6) and four (TRPM 1, 2, 5, 7) members, respectively [42].
- The presence of members of all three TRP channels has been demonstrated in leucocytes and leucocytic cell lines, with mRNA for TRPC6 having been demonstrated in human neutrophils, eosinophils and lymphocytes [42]. In addition, the ADP-ribose-activated long TRP channel 2 (LTRP 2) is expressed on neutrophil membranes and patch-clamp electrophysiological studies have demonstrated Na^+ and Ca^{2+} ion conductivity across these channels [43].
- Overexpression of TRPs in mammalian cells has been reported to result in enhancement of store-operated Ca^{2+} entry in many, but not all studies [41,44], while reduction of TRP expression using antisense strategies has been shown to decrease store-operated uptake of the cation [41].
- As described below, filling of intracellular Ca^{2+} stores through TRPs may involve physical interaction of the channel proteins with IP_3 receptors on storage vesicles, compatible with a conformational coupling mechanism of depletion-activated Ca^{2+} entry [41,45].

Although store-depletion activated mechanisms predominate during the refilling of intracellular Ca^{2+} stores, other pathways for Ca^{2+} entry may exist as chemokines utilizing CXC4, CCR1 and CCR5 on neutrophils are able to stimulate Ca^{2+} influx without Ca^{2+} release from storage vesicles [46].

Interestingly, in HEK 293 cells, a physical association has been reported to exist between TRPC3 channels and the Na^+/Ca^{2+} exchanger, with the TRPC3-mediated Ca^{2+} entry being dependent on the exchanger operating in reverse mode [47], as has been reported for neutrophils [37].

How do calcium storage vesicles 'communicate' with plasma membrane store-operated calcium channels?

Several mechanisms have been proposed to explain the activation of Ca^{2+} entry by store depletion and these have been the topic of several recent reviews [41,48].

Briefly, the conformational coupling model proposes that intracellular Ca^{2+} stores are located in close proximity to the plasma membrane, enabling physical coupling between the IP_3 receptors of the stores and the proteins of the Ca^{2+} entry channels. When the IP_3 receptors open during intracellular Ca^{2+} mobilization, the resultant conformational change in the receptor protein activates the Ca^{2+} entry channels, leading to Ca^{2+} influx [41,45]. In human neutrophils, the magnitude of influx of Ca^{2+} appears to be related directly to the intracellular IP_3 concentration [49].

The soluble intracellular messenger, calcium influx factor (CIF), may diffuse from the cytosol to activate plasma membrane calcium channels, triggering Ca^{2+} entry. The bioactive sphingolipid, sphingosine-1-phosphate, has been reported to mimic closely the actions of CIF [50], but this remains to be established conclusively [51].

More recently, additional mechanisms have been proposed. These include (1) a role for mitochondria in Ca^{2+} signalling [52], (2) participation of the cytoskeleton during calcium influx [53], (3) a direct mass-action mechanism [54] and (4) calcium-sensing proteins (CALPs) that may modulate the activity of calcium channels [55].

The various mechanisms utilized by chemoattractant-activated neutrophils to mobilize intracellular Ca^{2+} and to restore Ca^{2+} homeostasis are summarized in Fig. 2.

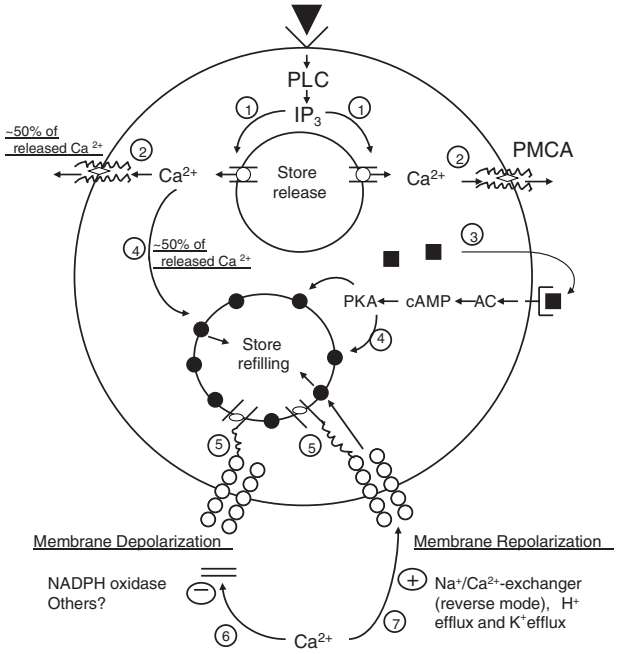

Fig. 2. A model of the mechanisms used by N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated human neutrophils to mobilize Ca^{2+} from intracellular stores, and to restore Ca^{2+} homeostasis. Interaction of the chemoattractant (▼) with G-protein/phospholipase C (PLC)-coupled membrane receptors (V) results in generation of inositol-1,4,5-triphosphate (IP_3) which, in turn, activates release of Ca^{2+} from intracellular stores 1 . The resultant transient elevation in cytosolic Ca^{2+} precedes, and is a prerequisite for, activation of several important proinflammatory activities of the cells. Clearance of the cation from the cytosol is achieved in part by efflux via the plasma membrane Ca^{2+}-ATPase (PMCA) 2 . Activation of the cells is also accompanied by synthesis and release of adenosine (■, 3) which interacts with plasma membrane adenosine subtype A_{2A} receptors (–[) coupled to adenylate cyclase (AC) and activation of cAMP-dependent protein kinase A (PKA). PKA in turn up-regulates the endomembrane Ca^{2+}-ATPase (●) on intracellular Ca^{2+} stores, leading to resequestration of the cation 4 . Refilling of stores is also mediated through uptake of extracellular Ca^{2+} via store-operated channels which are thought to be coupled to the IP_3 receptor 5 , with uptake of the cation being antagonized by the membrane depolarizing actions of NADPH-oxidase 6 , and facilitated by the repolarizing actions of electrogenic ion transporters such as the Na^+, Ca^{2+}-exchanger, and H^+ and K^+ efflux 7 .

Ca^{2+} homeostasis as a target for neutrophil-directed anti-inflammatory chemotherapy

Recent insights into the mechanisms utilized by neutrophils to restore Ca^{2+} homeostasis following activation with chemoattractants have enabled the identification of novel, potential targets on these cells for anti-inflammatory therapeutic agents. These targets, as well as the pharmacological strategies which may be used to achieve anti-inflammatory effects, are shown in Table 2.

Cyclic AMP-elevating agents

Cyclic AMP-elevating agents have been reported to interact directly with immune and inflammatory cells, including neutrophils, resulting in cAMP-mediated attenuation of the responses of these cells to various proinflammatory stimuli [56]. Importantly, these agents have been demonstrated to inhibit the activity of phospholipase A_2, as well as the production of a range of proinflammatory mediators including cytokines (especially tumour necrosis factor), prostaglandins, leukotrienes, PAF, reactive oxidants and release of granule-derived enzymes. Cyclic AMP-elevating agents also attenuate β_2-integrin activation/expression by neutrophils, eosinophils, monocytes, macrophages and lymphocytes [56,57].

Antagonists of depletion-activated store-refilling in neutrophils

Interference with the refilling of depleted Ca^{2+} stores in activated neutrophils represents an attractive target for anti-inflammatory chemotherapy, because this would be expected to prevent activation of the proinflammatory activities of the cells on re-exposure to the same or a different receptor-

Table 2. Mechanisms involved in mobilization of Ca^{2+} and restoration of Ca^{2+} homeostasis as potential targets for neutrophil-directed anti-inflammatory chemotherapy.

Target	Function	Pharmacological anti-inflammatory strategy	Examples of pharmacological agents/antagonists
Phospholipase C	Ca^{2+} mobilization	Inhibit: reduces Ca^{2+} mobilization	U73122
Plasma membrane Ca^{2+}-ATPase	Ca^{2+} clearance	Potentiate: down-regulates proinflammatory activities	Acidic phospholipids Polyunsaturated fatty acids
NADPH oxidase	Exclusion of extracellular Ca^{2+}	Potentiate: prevents store refilling and reactivation of cells	Clofazimine
Endomembrane Ca^{2+}-ATPase	Ca^{2+} clearance	Potentiate: down-regulates proinflammatory activities	β-Adrenoreceptor agonists Phosphodiesterase inhibitors Adenosine receptor agonists
Na^+/Ca^{2+}-exchanger	Facilitates uptake of Ca^{2+}	Inhibit: prevents store refilling and reactivation of cells	KB-R7943 SEA0400
Store-operated Ca^{2+} channels	Mediates uptake of Ca^{2+}	Block: prevents store-refilling and reactivation of cells	Itraconazole

dependent mediator of Ca^{2+} mobilization. Calcium reuptake by stimulated neutrophils increases the cytosolic Ca^{2+} concentration near the plasma membrane which facilitates degranulation and calpain activation [52], as well as β_2-integrin-mediated adhesion to vascular endothelium [58]. Antagonists of calcium influx may therefore attenuate these neutrophil responses. Agents which fall into this category include, first, those which inhibit the membrane repolarization responses of activated neutrophils, particularly inhibitors of the Na^+, Ca^{2+}-exchanger, and secondly, antagonists of store-operated Ca^{2+} channels such as itraconazole [59].

The therapeutic potential of anti-inflammatory strategies directed at Ca^{2+} metabolism

Beta-adrenergic agonists

Beta-adrenergic agonists (β-agonists) bind to β-receptors on inflammatory cells, including neutrophils, with resultant elevation of intracellular cAMP and inhibition of the proinflammatory activities of these cells *in vitro* [60,61]. In the clinical setting, numerous trials have supported the use of long-acting β-agonists (formoterol and salmeterol) in conjunction with inhaled corticosteroids, for patients with asthma and COPD [62–64].

Currently, the therapeutic potential of β-agonists, at clinically relevant concentrations, in experimental models of acute lung injury (ALI) [65] and in critically ill patients with the acute respiratory distress syndrome (ARDS) has generated interest [66,67].

Second-generation type 4 phosphodiesterase (PDE) inhibitors

PDE is a generic term that encompasses at least 11 distinct enzyme families (isotypes) that hydrolyse cAMP and/or cGMP, representing the only cellular pathway for the degradation of these cyclic nucleotides. Cyclic AMP-specific PDE4 is present in most immune and inflammatory cells, with the 4B2 subtype being the predominant PDE species in human neutrophils and monocytes [68,69]. Consequently, there has been considerable interest in PDE4-selective inhibitors as a potential therapy for acute and chronic inflammatory diseases, including ALI, bronchial asthma and COPD, and these agents have indeed been shown to exert significant anti-inflammatory activity in various animal models [70–72] and in clinical trials in humans [73], several of which are currently in progress. These involve compounds designed to have an improved therapeutic window such as cilomilast [*c*-4-cyano-4-(3-cyclopentoxyl-4-methoxyphenyl-*r*-1-cyclohexane carboxylic acid)], also known as Ariflo™ or SB 207499, which has been shown to improve pulmonary functions and symptoms in patients with COPD [74]. Importantly, the anti-inflammatory effects of cilomilast have been confirmed in patients with COPD in whom this agent significantly decreased the numbers of inflammatory cells determined by means of serial bronchial biopsies [75].

Another selective PDE4 inhibitor, roflumilast (3-cyclopropylmethoxy-4-difluoromethoxy-N-[3,5-di-chloropyrid-4-yl]benzamide), inhibits neutrophil proinflammatory responses *in vitro* [76] and has also demonstrated efficacy in the management of exercise-induced asthma [77] and COPD [73].

Adenosine receptor agonists

The well-recognized broad-spectrum anti-inflammatory actions of adenosine and its analogues are for the most part mediated through activation of the $A_{2A}R$ subtype. This is certainly the case for neutrophils, monocytes, mast cells and T lymphocytes [30,78]. The $A_{2A}R$ subtype is a G-protein (G_s)-coupled receptor linked to activation of adenylyl cyclase,

resulting in cAMP-mediated anti-inflammatory activity [79,80].

The prototype $A_{2A}R$ agonist, CGS 21680 (2(4-[(2-carboxyethyl)phenyl] ethylamino)-5′-N-ethylcarboxamido adenosine), although lacking the required receptor specificity for therapeutic application, together with ZM 241385 (4-(2-[7-amino-2-(2-fury 1)[1,2,4)triazolo[2,3-a][1,3,5]triazin-5-yl aminoethyl phenol), a highly selective antagonist of $A_{2A}Rs$, have been useful in probing the presence and anti-inflammatory properties of $A_{2A}Rs$ in immune and inflammatory cells [81,82]. CGS 21680 has also proved useful in the development of novel, experimental $A_{2A}R$ agonists with improved receptor specificity and anti-inflammatory properties [79,83].

The anti-inflammatory potential of adenosine, adenosine A_{2A} receptor agonists, adenosine precursors and enzyme inhibitors which elevate adenosine levels holds promise in the management of a range of inflammatory disorders including asthma, sepsis, auto-immune diseases, myocardial ischaemia and reperfusion injury [82,84–86]. The adenosine $A_{2A}R$ agonists, CGS 21680 and 4-[3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl]-cyclohexanecarboxylic acid methyl ester (ATL146e), exhibited significant anti-inflammatory activity in animal models of allergic asthma [87] and sepsis [88], respectively. During clinical trials in patients with acute myocardial infarction, adenosine significantly reduced infarct size [89]. Potent A_{2A} receptor agonists are currently being developed and the efficacy of these agents in the treatment of COPD remains to be established [90]. The widespread anatomical distribution of $A_{2A}Rs$ increases the potential for side effects, but this may be overcome by selective topical application in the lung, or by combining $A_{2A}R$ agonists with selective PDE4 inhibitors, or corticosteroids, which may enable reductions in the dosages of both types of agent [30,91].

Inhibitors of the Na^+/Ca^{2+}-exchanger

KB-R7943 has proved to be a useful experimental agent to probe the involvement of the Na^+, Ca^{2+}-exchanger in regulating the intracellular Ca^{2+} concentration in a variety of cell types such as ventricular myocytes, as well as the validity of the exchanger as a potential target for the prevention and therapy of ischaemia-related cardiovascular damage and dysfunction [92]. SEA0400 (2-[4,2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline), is a recently developed compound that appears to be a more potent and selective inhibitor of the Na^+/Ca^{2+}-exchanger than KB-R7943, and has been reported to be efficacious in animal models of cerebral ischaemic injury [92,93].

The anti-inflammatory potential of inhibitors of the Na^+/Ca^{2+}-exchanger, while extremely promising is, however, a very new area of immunopharmacological research. Meaningful progress will be dependent on identifying the isoform(s) of the exchanger which are operative not only in human neutrophils, but also in other types of immune and inflammatory cells [94,95], as well as on the design and development of isoform-specific inhibitors of the exchanger which selectively target these cells.

Antagonists of store-operated Ca^{2+} channels

As mentioned earlier, there is considerable interest in store-operated Ca^{2+} channels as potential therapeutic targets [42]. The imidazole antimycotic, itraconazole, antagonizes store-operated Ca^{2+} influx mechanisms in human neutrophils *in vitro* [59], while the structurally related triazole, fluconazole, improved survival in a limited number of critically ill patients with septic shock, an effect not attributed to its anti-fungal activity [96]. Clearly, selective and potent inhibitors of the TRP channel family have therapeutic potential for a range of inflammatory disorders. Although this field of immunopharmacology is exciting and holds considerable therapeutic promise, meaningful progress is, however, dependent on target validation and on the design and synthesis of selective pharmacological agents.

Phospholipase C (PLC) and NADPH oxidase

U73122, an inhibitor of PLC, has been shown *in vivo* to block carrageenan-induced paw oedema and leucocyte accumulation, as well as lipopolysaccharide-induced cellular infiltration and prostaglandin production in animal models [97]. Clofazimine, an antileprosy agent, potentiates NADPH oxidase activity [98], but also possesses well-documented anti-inflammatory actions [99], possibly reflecting the recognized role of the oxidase in restraining Ca^{2+} influx [22,35,37,100].

Conclusions

Recent insights into the mechanisms utilized by activated neutrophils to restore Ca^{2+} homeostasis have identified potential novel targets for anti-inflammatory chemotherapy. Foremost among these is the Ca^{2+} sequestering/resequestering, cAMP-up-regulated endomembrane Ca^2 ± ATPase which is amenable to selective pharmacological manipulation by second generation inhibitors of type 4 PDE, as well as by selective agonists of subtype $A_{2A}Rs$. Other targets which hold promise include the Na^+/Ca^{2+}-exchanger and Ca^{2+} uptake channels of the TRP family.

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